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Award Number: W81XWH-04-1-0480

TITLE: An Organotypic Liver System for Tumor Progression

PRINCIPAL INVESTIGATOR: Alan Wells, M.D. D.M.Sc.
Linda Griffith, Ph.D.
Donna Stolz, Ph.D.
Douglas Lauffenburger, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, PA 17213

REPORT DATE: April 2007

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-04-2007	Final Addendum	1 Apr 2006 – 31 Mar 2007

4. TITLE AND SUBTITLE

An Organotypic Liver System for Tumor Progression

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0480

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Alan Wells, M.D. D.M.Sc.
Linda Griffith, Ph.D.
Donna Stolz, Ph.D.
Douglas Lauffenburger, Ph.D.
Email: wellsa@upmc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Pittsburgh
Pittsburgh, PA 17213

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Our overall objective is to understand which tumor cell behaviors contribute to invasion and metastasis. This would allow rationale approaches to limit these aspects of tumor progression. While great strides have defined critical molecular determinants, the current experimental models of tumor invasion limit the dissection of complex cellular responses. In vitro assays do not capture tumor/host relations or relevant tissue architecture and physiology. In vivo model systems provide the relevant organism contexts but cannot readily be manipulated. Quantal advances would be enabled by combining the best attributes – direct manipulation of tumor and host, long-term visualization, and tissue relevant architecture. Our central premise is that an ex vivo organotypic liver tissue system can provide an environment to study tumor cell invasion and metastasis. Our objective is to utilize a physiologically relevant microreactor that has proved suitable for organotypic liver culture to investigate metastatic seeding. The sub-millimeter scale of this liver allows for real-time imaging over weeks in culture. We established this system to determine what step is rate-limiting for tumor progression. We have now established an organotypic liver tissue culture that supports metastatic establishment and growth. This will be used to probe the molecular steps that are key to this progression.

15. SUBJECT TERMS
Tumor metastasis, novel technology, liver, bioreactor

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	18	19b. TELEPHONE NUMBER *(include area code)*

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AN ORGANOTYPIC LIVER SYSTEM FOR TUMOR PROGRESSION

Alan Wells, Linda Griffith, Donna Stolz, Douglas Lauffenburger

INTRODUCTION

Our overall objective is to understand which tumor cell behaviors contribute to invasion and metastasis. This would allow rationale approaches to limit these aspects of tumor progression. While great strides have defined critical molecular determinants, the current experimental models of tumor invasion limit the dissection of complex cellular responses. *In vitro* assays such as transmigration of barrier matrices or cell layers allow for targeted perturbations, but do not capture tumor/host relations or relevant tissue architecture and physiology (1). *In vivo* model systems, mainly xenografts and induced tumors, provide the relevant organism contexts but cannot readily be manipulated. Recent advances in imaging of tumors in living animals aids in documenting events, though these views still occur only over hours and only near the surface of observation (2). Thus, quantal advances would be enabled by new assay systems that combine the best attributes of both – direct manipulation of tumor and host, long-term (days to weeks) visualization, and tissue relevant architecture.

Our central premise is that an ex vivo organotypic liver tissue system can provide an environment to study tumor cell invasion and metastasis. Our objective is to utilize a physiologically relevant microreactor that has proved suitable for organotypic liver culture (3) to investigate cellular and molecular events during tumor metastatic seeding. The sub-millimeter scale of this liver allows for real-time imaging throughout the entire tissue over weeks in culture. We propose to use this system to determine when and how motility is rate-limiting for tumor progression. The first step in gaining this capability, and the one supported by the DoD funds is to determine whether an organotypic liver tissue culture supports metastatic establishment and growth.

BODY

The revised and accepted Statement of Work (Table 1) described a series of tasks to accomplish the one accepted Objective. We tackled these Tasks in the order of greatest yield so that work in areas progressed as systems were optimized in others. During the three year time frame of this grant we have accomplished these tasks.

Table 1. Statement of Work

<u>Work to be performed at University of Pittsburgh (Wells and Stolz Laboratories):</u>

1. isolate hepatocytes and endothelial cells – *completed*
2. label tumor cells – *completed*
3. seed bioreactors with cells – *completed*
4. seed organotypic liver bioreactors with tumor cells – *completed*

<u>Work to be performed at MIT (Griffith and Lauffenburger Laboratories):</u>

5. design bioreactor scaffolds for high volume production – *completed*
6. optimize new bioreactor for continuous two-photon imaging – *completed*
7. produce bioreactor scaffolds – *completed*
8. deconvolute images to determine tumor-hepatocyte involvement and growth dynamics – *in progress*

<u>Additional tasks accepted after Year 1:</u>

9. determine whether breast cancer cell E-cadherin form heterotypic interactions – *completed*
10. determine whether hepatocytes modulate cancer cell E-cadherin expression – *completed*

Work to be performed at University of Pittsburgh:

Task 1. Isolate hepatocytes and endothelial cells. This task has been established. We have been successfully isolating both hepatocytes and endothelial cells as viable cells. These have been isolated both from wild-type rats and from GFP-expressing rats. These have been incorporated into the pre-bioreactor spheroids (Figure 1). We routinely obtain over 80% viability in these preparations. This is sufficient for generating the organotypic culture system.

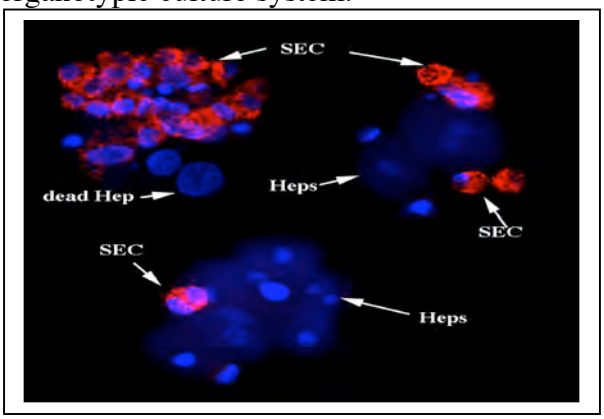


Figure1. The rat endothelial cells (vitally stained red by di-I uptake, and labelled SEC in figure) are alive within the hepatocyte spheroids (nuclei of all cells stained blue by DAPI; hepatocytes are identified by morphology and labelled heps) prior to loading of the bioreactor.

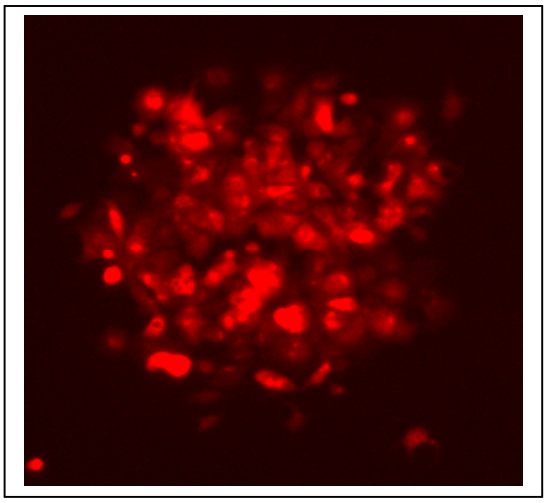
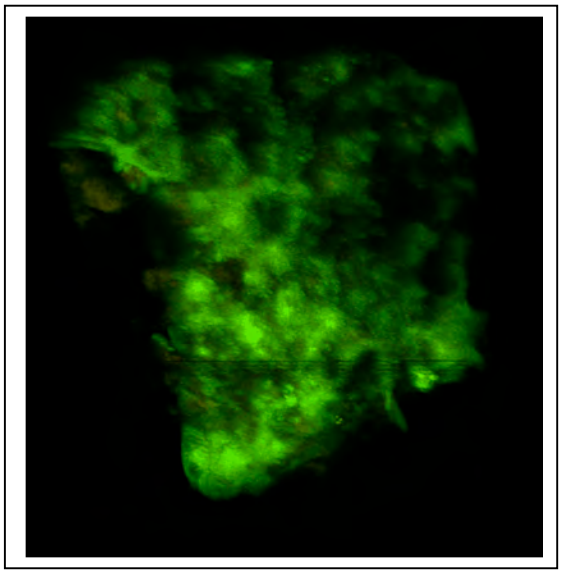


Figure 2. MCF7 breast cancer cells fluorescencing red from stable expression of RFP. RFP-expressing cells were selected by flow cytometry and passaged in culture for over 3 weeks. Virtually all cells express fluorescence demonstrating feasibility of following these cells for up to a month in the bioreactor.

Task 2. Label tumor cells. We established MCF7, MDA-468 and MDA-231 cells stably expressing RFP in addition to GFP (Figure 2a). In order to track primary breast cancer cells, we needed a method to label these cells. Stable transfection would not be consistent with maintaining these cells in early passage and heterogeneity. We used cell tracker dyes to label these cells (Figure 5). These breast cancer cells are in addition to the prostate cancer cells that we labeled previously (Yates et al., 2007).



Task 3. Seed bioreactors with cells. This task has been fully established. We have generated liver bioreactors with transgenic hepatocytes and/or endothelial cells expressing GFP to better image the interactions with the tumor cells. These cells form bioreactor structures indistinguishable from non-transgenic cells (Figure 3). As the hepatocytes have an endogenous fluorescence in the same channel as GFP, this interfered with imaging tumor cells in this channel. As such, we have switched our tumor cell imaging to the red channel with RFP.

Figure 3. The hepatocytes and non-parenchymal cells from the GFP-transgenic rats establish channel-filled organoid cultures in the bioreactor similar to non-GFP-transgenic animal cells, as shown here 5 days after seeding.

Task 4. Seed organotypic liver bioreactors with tumor cells. This task is fully established. We have accomplished this task with breast and prostate tumor cell lines (Figure 4) and primary cells (Figure 5). The tumor cells proliferate over a few day period to eventually take over the entire bioreactor. Of interest, individual tumor cells intercalate between the hepatocytes and appear to form tight contacts with them (Figure 6) The newer bioreactor scaffold are higher volume production also enable better histological and electron microscopic examinations (Figure 7).

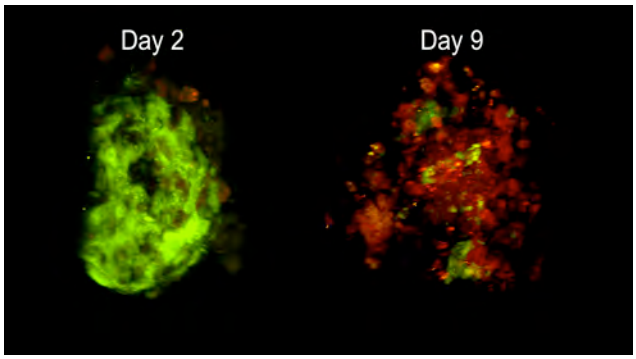


Figure 4. The RPF-labelled tumor cells expand from a few isolated cells (red) among the hepatocyes (green) noted 2 days after seeding the bioreactor (left image), to form the predominant cell mass a week later (right image). Shown are images from the identical bioreactor channel; these are representative of over 50 total channels in at least 6 independent experiments.

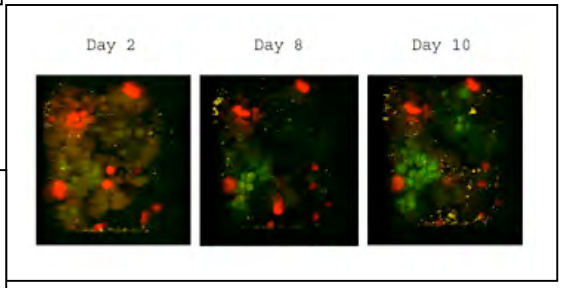


Figure 5. Primary breast cancer cells were obtained as a gift from Precision Therapeutics Inc. These were dyed with red cell tracker and inoculated into the liver organotypic bioreactor.

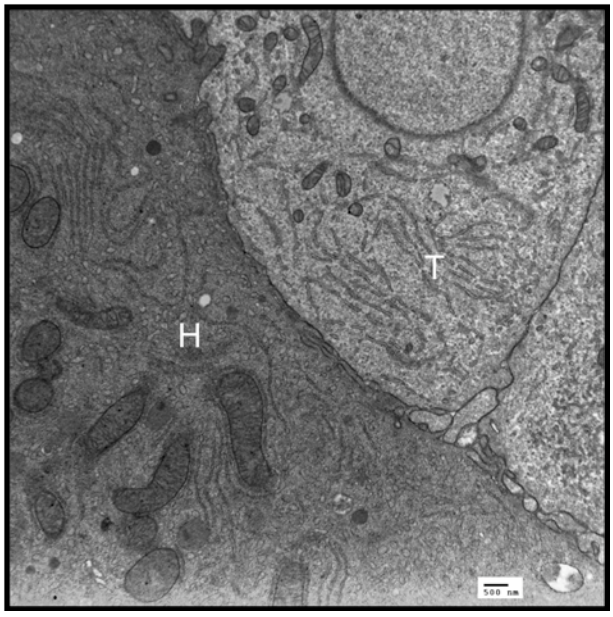
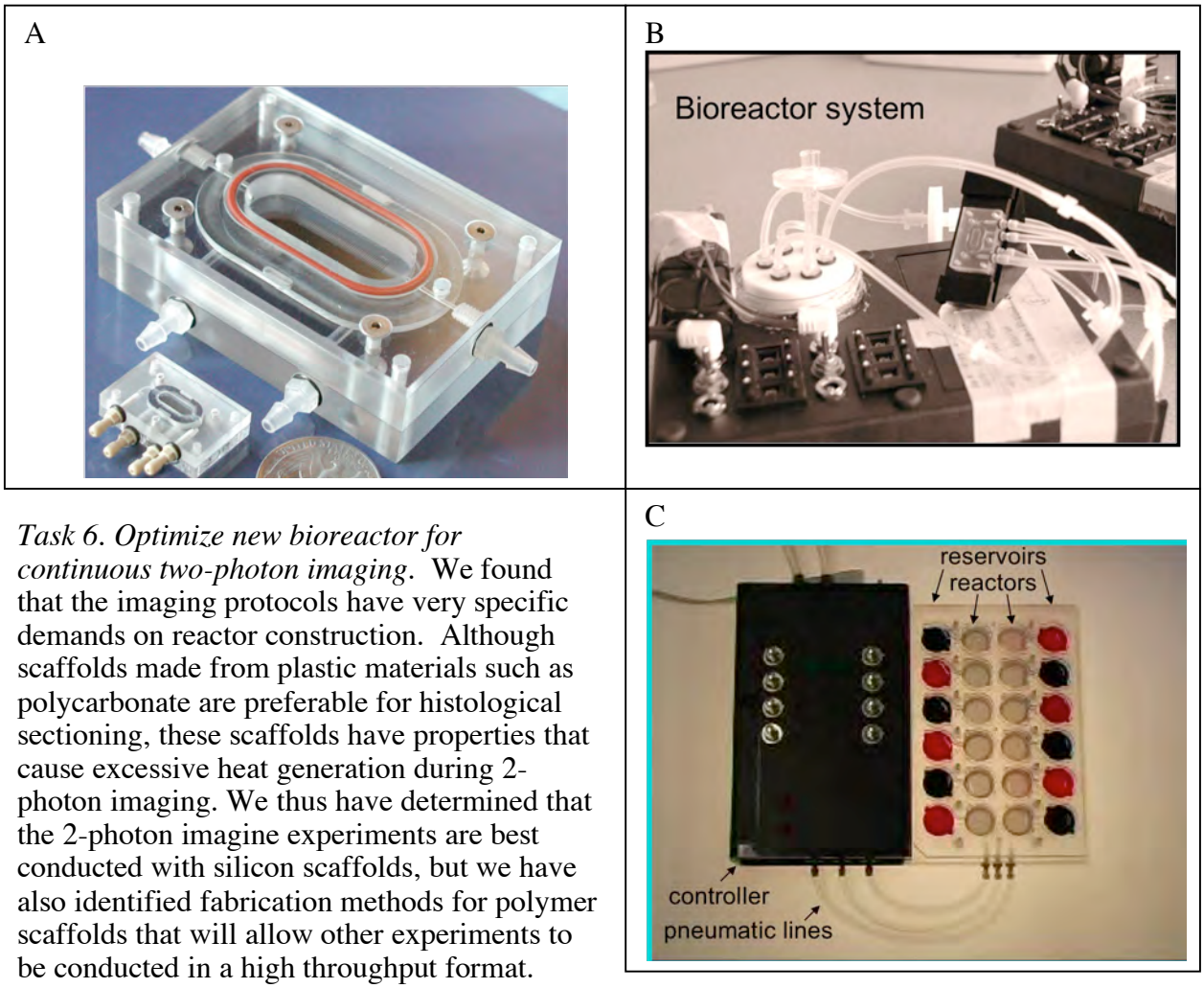


Figure 6. Transmission electron micrograph of tumor cell (T) that is juxtaposed to a hepatocyte (H) in the bioreactor. The invaginations of the membranes at the interface between the two cells are indicative of close communication between the two cells.

<u>*Work to be performed at MIT:*</u>

Task 5. Design bioreactor scaffolds for high volume production. The original bioreactor format was designed for in situ 2-photon imaging and requires a substantial technical effort to seed and run each individual reactor. This approach may be acceptable for 2-photon imaging, but there other assays of interest that are desirable to perform on a high-throughput basis in a multi-well format, including assays of cell growth rate, influence of various non-parenchymal cell types on tumor behavior, and histology. Therefore we developed a multi-well plate format version of the bioreactor based on microfluidic pumping (Figure 7). The current prototype has 12 bioreactor chamber wells in a standard 24-well plate footprint and can be loaded with cells by simple pipetting. The liver function in this system is comparable to that in the original reactor, using a panel of PCR probes for key P450 enzymes and liver-enriched transcription factors. This has been accomplished during our no cost extension.

Figure 7. Three version of the bioreactor are currently used for studies. (a) the MilliReactor, which holds 50,000 cells and the Giant reactor, which holds 1.3 million cells (b) The millireactor in its fluidic circuit, showing the pumps and medium reservoir and battery power (c) the multi-well plate reactor, which can be scaled to hold 10,000 – 1,000,000 cells per well. In (c), colored fluids have been added to the medium reservoir to illustrate the fluid path.



Task 6. Optimize new bioreactor for continuous two-photon imaging. We found that the imaging protocols have very specific demands on reactor construction. Although scaffolds made from plastic materials such as polycarbonate are preferable for histological sectioning, these scaffolds have properties that cause excessive heat generation during 2-photon imaging. We thus have determined that the 2-photon imagine experiments are best conducted with silicon scaffolds, but we have also identified fabrication methods for polymer scaffolds that will allow other experiments to be conducted in a high throughput format. During our no cost extension we optimized these designs and now have functional scaffolds.

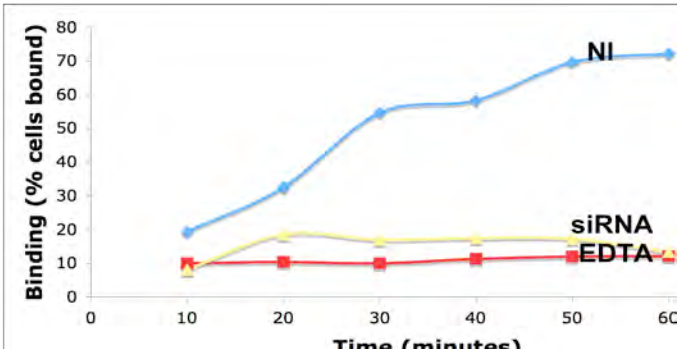
Task 7. Produce bioreactors and scaffolds for U. Pitt research. This is an ongoing task that generates sufficient bioreactors for studies. To-date, over 25 bioreactor scaffolds and assemblies have been delivered for use in these studies outlined herein. We fabricated silicon scaffolds using deep reactive silicon etching at MIT, and produce these on a semi-high throughput basis. We investigated several approaches to fabricating scaffolds from polymers to facilitate histological sectioning and to use in the multi-well plate format of the system. Approaches included laser machining of polycarbonate, polystyrene and polyimide sheets, injection molding of polypropylene, and micromachining of polycarbonate. Among these methods, micromachining of polycarbonate was the most promising, and and chosen for use. We are now ready to transfer the first multiwell bioreactor to U. Pitt, as the liver studies are completed.

Task 8. Deconvolute images to determine tumor-hepatocyte involvement and growth dynamics. The images of breast cancer cells in the bioreactor have been deconvoluted in four dimensions (through time). The cells have been seen to intercalate between the hepatocytes in the liver microtissue (Figure 5). The task was completed prior to our no cost extension.

Additional task

Based on preliminary finding of breast cancer cells juxtaposing to hepatocytes (Figure 6), we determined whether such cells can adhere to hepatocytes via E-cadherin homotypic binding. This is listed as a change in the Year 1 report which was approved. This has been accomplished for MCF7 cells which express E-cadherin (Figure 8). MCF7 bound to hepatocytes in a E-cadherin-dependent manner as EDTA, which chelates calcium eliminating E-cadherin homotypic binding, and siRNA to E-cadherin eliminated this association.

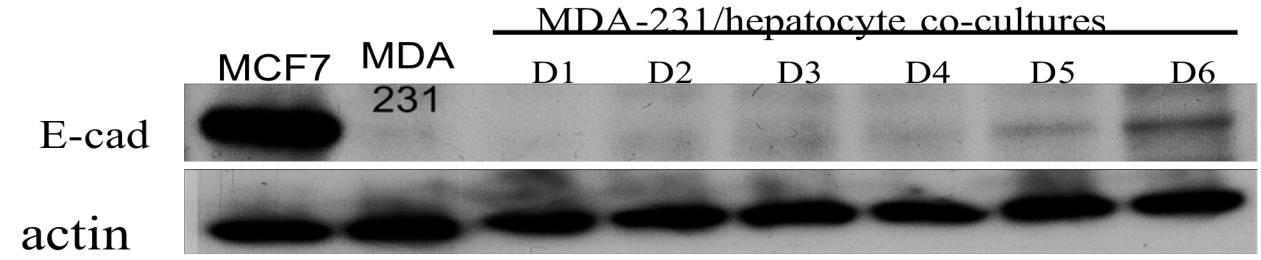
Figure 8. MCF-7 breast cancer cells that express E-cadherin form heterotypic binding to a monolayer of hepatocytes as determined by centrifugal assay for cell adhesion (CAFCA) (NI). This is due to E-cadherin as it is disrupted by the calcium chelator EDTA (EDTA) and siRNA that downregulates E-cadherin by >70% (siRNA).



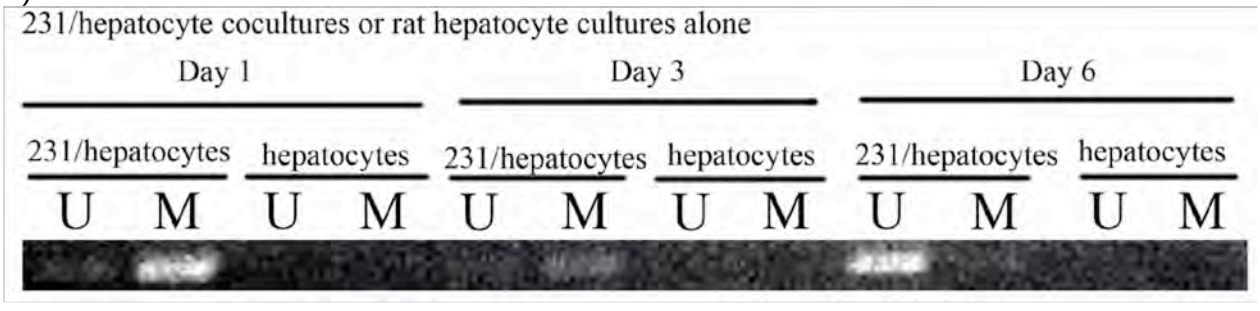
During the no cost extension, we examined E-cadherins on aggressive breast cancer cells that have transcriptional silencing of their E-cadherin promoter. We found that upon co-culture with hepatocytes, MDA-231 cells re-express their E-cadherin concomitant with loss of promoter methylation specific to the E-cadherin promoter (Figure 9).

Figure 9. Coculture of aggressive human breast carcinoma cells, MDA-231 with primary rat hepatocytes leads to E-cadherin upregulation at the protein level (A) concomitant with loss of E-cadherin promoter methylation (B). (A) Cells were cocultured in serum-free media for up to 6 days with increase in E-cadherin by immunoblot using a human-specific antibody. (B) Methylation-specific PCR detects a shift from methylated (M) to unmethylated (U) over this same time period; a constitutively methylated promoter is not affected (not shown).

A)



B)



KEY RESEARCH ACCOMPLISHMENTS

- A liver bioreactor can support growth of tumor cells even in the absence of serum
- Primary human tumor explants can be grown in this bioreactor environment in the absence of serum
- The combined liver/tumor bioreactor is viable over 28 days
- The liver bioreactor contains both parenchymal (hepatocytes) and nonparenchymal (endothelial and stromal cells) consistent with the metastatic environment
- The liver bioreactor can be made to be high throughput
- Tumor cells intercalate between hepatocytes as individual cells
- Tumor cells in the bioreactor show evidence of tight junctions with the hepatocytes
- Breast carcinoma cells can form functional cell heterotypic interactions with hepatocytes
- Hepatocytes can direct aggressive breast cancer cells to re-express E-cadherin

REPORTABLE OUTCOMES

Articles:

C Yates, CR Shepard, G Papworth, A Dash, **DB Stolz**, S Tannenbaum, **L Griffith, A Wells** (2007). Novel three-dimensional organotypic liver bioreactor to directly visualize early events in metastatic progression. Advances in Cancer Research 97, in press.

CC Yates, CR Shepard, **D Stolz, A Wells** (2007). Co-culturing human prostate carcinoma cells with hepatocytes leads to increased expression of E-cadherin. British Journal of Cancer 96, 1246-1252.

Abstracts:

C Yates, **D B Stolz, L Griffith, A Wells** (2004) Direct Visualization of Prostate Cancer Progression Utilizing a Bioreactor. American Association for Cancer Research Annual Meeting, Orlando, FL oral presentation

C Yates, **D B Stolz, L Griffith, A Wells** (2005) Ex Vivo Metastasis Model for Prostate Cancer Progression. Regenerate 2005, Altanta, GA oral presentation

L Grifith, C Yates, A Sivaraman, K Domansky, **D B Stolz,** S R Tannenbaum, **A Wells** (2005) Microscale Tissue Engineering of Liver, 20th Aspen Cancer Conference, Aspen, CO oral presentation

A Dash, **L Griffith, A Wells,** S R Tannebaum (2005) Activation of c-Met in Hepatocyte-Prostate Cancer Cell Co-cutlures, 20th Aspen Cancer Conference, Aspen, CO poster presentation

C Yates, **D B Stolz, L Griffith, A Wells** (2005) Characterization of Prostate Cancer Progression by Direct Visualization Utilizing a Bioreactor. American Association for Cancer Research Annual Meeting, Anaheim, CA oral presentation

C Yates, **D B Stolz, L Griffith, A Wells** (2005) Direct Visualization of Prostate Cancer Progression Utilizing an Organotypic Liver Bioreactor. Academy of Clinical Laboratory Physicians and Scientists Annual Meeting, Pittsburgh, PA oral presentation

C Yates, **D B Stolz, L Griffith, A Wells** (2005) An Organotypic Liver Bioreactor as a Metastasis Model for Tumor Progression. Regenerate 2005, Atlanta, GA poster

L Grifith, C Yates, A Sivaraman, K Domansky, **D B Stolz,** S R Tannenbaum, **A Wells** (2005) In Vitro Physiological Tissue Models for Drug Discovery, Emory/Georgia Tech Research Institute for Predictive Health, Predictive Health Symposium oral presentation

L Grifith, C Yates, A Sivaraman, K Domansky, **D B Stolz,** S R Tannenbaum, **A Wells** (2006) Human Body on A Chip: Microscale Tissue Engineering for Drug Discovery, New York Academy of Sciences, Predictive Toxicology Symposium oral presentation

L Grifith, C Yates, A Sivaraman, K Domansky, **D B Stolz,** S R Tannenbaum, **A Wells** (2006) Human Body on A Chip: Microscale Tissue Engineering of Liver and Bone Marrow for Drug

Discovery, Boston Discovery Toxicology Meeting, Cambridge, MA oral presentation

C Yates, C Shepard, **A Wells** (2006) A Critical role of E-cadherin expression during the Progression of Metastatic Prostate Cancer cells with the Microenvironment as a Driving Force. American Association for Cancer Research Annual Meeting, Washington, DC oral presentation

CR Shepard, **A Wells** (2006) Re-expression of E-cadherin by Invasive Breast Cancer Cells as a Strategy for Metastatic Colonization of the Liver, San Antonio Breast Cancer Syposium. San Antonio, TX plenary session (oral presentation)

CR Shepard, **A Wells** (2007) Demethylation of the E-cadherin Promoter Driven by Hepatocytes Allows of Cell Fate-Determining Signals in Invasive Breast Cancer Cells. Experimental Biology 2007, Washington, DC highlights in graduate student research in pathology (oral presentation)

Book chapters:

C Yates, **DB Stolz, LG Griffith** (2005) Imaging Invasion and Metastasis ex vivo. In Cell Motility in Cancer Invasion and Metastasis (Ed: A Wells, Kluwer Academic Press).

A Wells (2005). Motility in tumor invasion and metastasis – an overview. In Cell Motility in Cancer Invasion and Metastasis (Ed: A Wells, Kluwer Academic Press).

CONCLUSIONS

This award has reached defined milestones including the additional tasks added after Year 1. As originally funded by the DoD, we have generated a metastasis model system for mechanistic investigations in breast cancer. Initial functional studies using this model have also highlighted new directions for future research in that we find close contacts between hepatocytes and tumor cells and that hepatocytes can drive E-cadherin re-expression.

Importance/Implications: The Key Accomplishments above firmly demonstrate the ability to use this novel model system for human metastasis. This provides the 'proof a concept' that such a model system can examine intermediary scale events during metastatic growth. Further, the finding of close heterotypic cell interactions highlights new avenues for study.

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2. J Condeelis, J E Segall (2003). Intravital imaging of cell movement in tumours. *Nature Reviews – Cancer* 3:921-930; J S Condeelis, J Wyckoff, J E Segall (2000). Imaging of cancer invasion and metastasis using green fluorescent protein. *European Journal of Cancer* 36:1671-1680; K J Luzzi, I C MacDonald, et al (1998). Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. *American Journal of Pathology* 153:865-873
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British Journal of Cancer (2007) 96, 1246–1252

www.bjcancer.com

Co-culturing human prostate carcinoma cells with hepatocytes leads to increased expression of E-cadherin

CC Yates[1], CR Shepard[1], DB Stolz[2] and A Wells*[,1]

[1]Department of Pathology, University of Pittsburgh, and Pittsburgh VAMC, Pittsburgh, PA 15213, USA; [2]Department of Cell Biology, University of Pittsburgh, and Pittsburgh VAMC, Pittsburgh, PA 15213, USA

Metastasis is a multi-step process wherein tumour cells detach from the primary mass, migrate through barrier matrices, gain access to conduits to disseminate, and subsequently survive and proliferate in an ectopic site. During the initial invasion stage, prostate carcinoma cells undergo epithelial–mesenchymal-like transition with gain of autocrine signalling and loss of E-cadherin, hallmarks that appear to enable invasion and dissemination. However, some metastases express E-cadherin, and we found close connections between prostate carcinoma cells and hepatocytes in a liver microtissue bioreactor. We hypothesise that phenotypic plasticity occurs late in prostate cancer progression at the site of ectopic seeding. Immunofluorescence staining for E-cadherin in co-cultures of hepatocytes and DU-145 prostate cancer cells revealed E-cadherin upregulation at peripheral sites of contact by day 2 of co-culture; E-cadherin expression also increased in PC-3 cells in co-culture. These carcinoma cells bound to hepatocytes in an E-cadherin-dependent manner. Although the signals by which the hepatocytes elicited E-cadherin expression remain undetermined, it appeared related to downregulation of epidermal growth factor receptor (EGFR) signalling. Inhibition of autocrine EGFR signalling increased E-cadherin expression and cell–cell heterotypic adhesion; further, expression of a downregulation-resistant EGFR variant prevented E-cadherin upregulation. These findings were supported by finding E-cadherin and catenins but not activated EGFR in human prostate metastases to the liver. We conclude that the term epithelial–mesenchymal transition only summarises the transient downregulation of E-cadherin for invasion with re-expression of E-cadherin being a physiological consequence of metastatic seeding.
British Journal of Cancer (2007) **96,** 1246–1252. doi:10.1038/sj.bjc.6603700 www.bjcancer.com
Published online 3 April 2007

Keywords: EGF receptor; centrifugal assay for cell adhesion; heterotypic cell–cell adhesion; epithelial–mesenchymal transition

Metastasis causes the vast majority of the morbidity and mortality from cancer. However, our understanding of the multi-step cascade of events that must occur for successful attachment and subsequent metastasis has not been completely elucidated, as few systems are available to examine the intermediary dynamic steps that occur during progression (Condeelis and Segall, 2003; Yates *et al*, 2007). Only now are investigators defining the changes needed for both a cell to escape from the primary tumour and subsequently to allow for ectopic survival and proliferation.

An essential step for cells to migrate from the primary tumour mass is the loss of epithelial cell–cell adhesions and subsequent acquisition of a mesenchymal-like migratory and invasive phenotype, generally referred to as epithelial–mesenchymal transition (EMT) (Thiery, 2002; Ackland *et al*, 2003; Bates and Mercurio, 2005). Central to this dissociation from the primary tumour is altered or loss of E-cadherin expression (Davies *et al*, 2000; Lowy *et al*, 2002) a key attribute that may define the EMT. E-cadherin is a calcium (Ca^{2+})-dependent transmembrane cell surface glycoprotein. E-cadherin rapidly localises on the cell surface to regions of contact, usually resulting in homotypic cell–cell binding; this fosters the maintenance of normal cellular structure. Recently, reports have shown that aberrant loss or downregulation of E-cadherin in carcinomas may result from reversible epigenetic events (Graff *et al*, 1995; Lind *et al*, 2004) or growth factor-mediated downregulation (Hurtubise and Momparler, 2004; Wheeler, 2005), suggesting that loss of this tumour suppressor may be reversible. That this reversal might happen has been suggested by the findings that secondary tumour metastases express E-cadherin (Brabletz *et al*, 2001; Rubin *et al*, 2001). However, that this expression at the distal site represents re-expression of E-cadherin has not been demonstrated.

Activation of the epidermal growth factor receptor (EGFR) downregulates E-cadherin expression (Jawhari *et al*, 1999; Ackland *et al*, 2003). This suggested that autocrine EGFR signalling present in prostate carcinomas may contribute to E-cadherin repression in these tumours. This was supported by our recent finding that pharmacological abrogation of EGFR signalling in prostate carcinoma cells reverses decreased E-cadherin expression rendering these cells less invasive and more cohesive (Jawhari *et al*, 1999; Yates *et al*, 2005). This re-expression of E-cadherin is similarly observed by close contacts of DU-145 prostate carcinoma cells when cultured within the liver microenvironment (Yates *et al*, 2007). As prostate cancer metastasises to the liver in over half of all patients with metastatic disease (Ewing, 1922; Shah *et al*, 2004), we sought to explore the correlative expression levels of EGFR and

*Correspondence: Dr A Wells; E-mail: wellsa@upmc.edu
Received 29 August 2006; revised 22 February 2007; accepted 22 February 2007; published online 3 April 2007

E-cadherin in the presence of parenchymal cells of the liver. We show, herein, that hepatocytes elicit E-cadherin expression in prostate carcinoma cells concomitant with downregulation of EGFR signalling.

MATERIALS AND METHODS

Materials

The primary antibodies used were mouse monoclonal antibodies selective for human E-cadherin, human cytokeratin 18 and human-specific controls tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Primary antibodies to phospho-EGFR (Cell Signaling, Danvers, MA, USA), α-catenin, β-catenin, p120 (BD Biosciences, San Jose, CA, USA) and vimentin (Dako Laboratories, Carpinteria, CA, USA). Polyclonal antibody to EGFR was used for immunohistochemistry (Santa Cruz). Secondary antibodies for immunofluorescence dyes or horseradish peroxidase-conjugated were obtained from (Jackson Laboratories, Bar Harbor, ME, USA). Anti-E-cadherin blocking antibody (SHE78-7) was obtained from Zymed Laboratories (South San Francisco, CA, USA). The EGFR inhibitor PD153035 and primary antibody to human EGFR (Ab-1), were obtained from Calbiochem (San Diego, CA, USA). Other reagents were obtained from Sigma (St Louis, MO, USA).

Cell lines

The androgen-independent DU-145 and PC-3 human prostate carcinoma cell line were originally derived from a brain and bone metastasis of a human prostate adenocarcinoma, respectively. A transmodulation-resistant EGFR construct was generated by replacing the PKC-target threonine at amino acid 654 with alanine (A654) and transfected into DU-145 cells (Yates *et al*, 2005). Prostate cancer cells lines were cultured as described previously (Yates *et al*, 2005). Red fluorescent protein (RFP) was stably transfected with ds-Red (Clontech, Mountain View, CA, USA) vector containing a neomycin resistance gene in DU-145 cells. RFP expressing cells were selected and maintained in DMEM with 10% FBS and in the presence of 1000 mg ml^{-1} G418 until used for experimentation.

Reverse transcription-PCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). RT reaction was performed using total RNA as a template and an RT for PCR kit (Invitrogen). PCR amplification was carried out with the following primers: human-specific E-cadherin primers, 5′-gacacccgattcaaagtggg-3′ and 5′-gtctctcttctgtcttctgag-3′; showed no homology to rat E-cadherin and 30.2% homology to mouse E-cadherin. GAPDH primer kit 5′-ccacccatggcaaattccatgg ca-3′ and 5′-tctaacggcaggtcaggtccacc-3′ (Stratagene, Cedar Creek, TX, USA) recognises both human and rodent sequences.

Hepatocytes

Primary rat hepatocytes, >90% purified, were obtained by collagenase perfusion (Seglen, 1976) of 150–230 g male eGFP (enhanced green fluorescent protein)-transgenic and WT (wild-type) Sprague–Dawley rats, originally generated by Dr Masaru Okabe (Genome Information Research Center, University of Osaka, Osaka, Japan) and were generously provided by Japan SLC, Inc. (Hamamatsu, Japan). Use of rat hepatocytes enables the identification of the human prostate carcinoma cell proteins using human-specific antibodies. The cells were collected and maintained in Hepatocyte Growth Media (HGM) medium before and during co-culture (Yates *et al*, 2007). These studies were approved by the University of Pittsburgh IACUC committee.

Co-cultures

Initial co-cultures consisted of 50 000 cells cm^{-2} of freshly isolated hepatocytes and 2000 cells cm^{-2} prostate cancer cells. Co-cultures were maintained in serum-free HGM (Yates *et al*, 2007), and plated on tissue culture dishes pre-treated with 10% collagen (Upstate, Charlottesville, VA, USA).

Centrifugal assay for cell adhesion

This assay is a modification of the McClay and Giacolmello assays (McClay *et al*, 1981). Cancer cells were non-enzymatically dissociated and labelled with 5 μM Calcein AM (Molecular Probes, Carlsbad, CA, USA). Labelled cancer cells were seeded at a density of 42 000 cells well^{-1} in 96-well plates containing a densely confluent hepatocyte monolayer. The plates were centrifuged for <60s at 50 **g** to pellet the cancer cells onto the hepatic monolayer, then incubated at 37°C. At defined times, the plates were inverted and centrifuged at 600 **g** for 5 min and then gently washed to remove unbound cells from the hepatocyte monolayer. Fluorescence was measured with a 494/517 bandpass filter set-up from the bottom of the plate by a TECAN Spectra-Fluor plate fluorometer. Absolute emission measurements were background subtracted.

Tissue specimens

Formalin-fixed paraffin-embedded tissues were obtained from the University of Pittsburgh Tumor Bank. We found only two well-defined prostate adenocarcinomas with liver metastases and none from the Cooperative Human Tumor Network, irrespective of other criteria. These studies, blinded for all personal identifiers, were categorised as exemption 4e by the University of Pittsburgh IRB.

Statistical analysis

Statistics for all experiments were performed using the Sigma Plot statistical program (Jandel Scientific, Chicago, IL, USA). Independent Student's *t*-test was utilised to determine a statistical difference between experimental and the controls for individual experiments, with significance generally informed at $P<0.05$.

RESULTS

E-cadherin is generally lost from the primary tumour cells of metastatic carcinomas, with the result that these cells can now detach from the primary tumour mass and attain a nonpolarised, migratory phenotype (Bates and Mercurio, 2005). Unlike many other tumour suppressors, the E-cadherin gene is neither deleted nor mutated, but rather the downregulation appears to result from epigenetic signals, opening the possibility that this loss of expression may be reverted later during the metastatic cascade (Yates *et al*, 2007). Intriguingly, E-cadherin expression has been noted on metastases of human carcinomas (Rubin *et al*, 2001). Thus, we proposed that E-cadherin re-expression coincided with distant metastases, enabling the prostate carcinoma cells to interact with the ectopic parenchyma (Yates *et al*, 2007). We tested this postulate using liver hepatocytes, as liver is the epithelial organ most commonly colonised by metastatic prostate carcinoma (Ewing, 1922; Shah *et al*, 2004).

We found that co-culturing DU-145 and PC-3 carcinoma cells with rat hepatocytes resulted in increased E-cadherin expression and decreased EGFR expression (Figure 1A). This occurred over a 6-day period in both cell types, with significant differences by day 2 (Figure 1A and B). The increase in E-cadherin protein levels was reflected by similarly elevated mRNA levels (Figure 1B), though the appearance of mRNA may lag by about

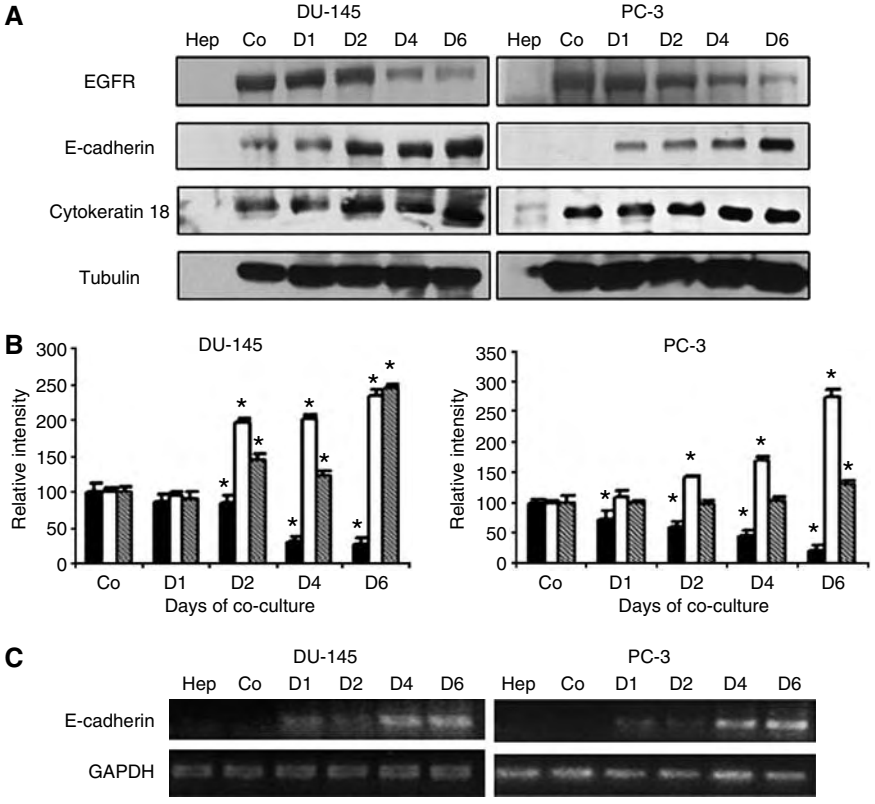

Figure 1 Co-culture of human prostate cancer cell with rat hepatocytes reversed E-cadherin expression. DU-145 or PC-3 (**A**) cells were co-cultured in the presence of primary rat hepatocytes over a 6-day period. Hepatocytes and single cultures were lysed before co-cultures. On days 1, 2, 4 and 6, co-culture lysates were immunoblotted with indicated human selective antibodies: anti-E-cadherin, anti-EGFR antibody, anti-cytokeratin 18 and anti-tubulin (as the loading control). (**B**) Densitometry of immunoblots in DU-145 and PC-3 cells co-cultures (■) EGFR, (□) E-cadherin () and Cytokeratin 18; shown are the mean ± s.d. of three blots with day 0 being at 100 (*$P<0.05$ from C0). Cellular levels of E-cadherin mRNA in DU-145 or PC-3 (**C**) cells were analysed by RT-PCR using GAPDH as a loading control. In **A** and **B**, Co (for control) are an equal number of prostate carcinoma cells incubated for 1 day in the absence of hepatocytes. The Hep (hepatocytes) were an equal number of hepatocytes as for the co-cultures. The first three lanes were all lysed at the same time. Shown are representative of at least three experiments.

1 day. This time differential may imply two levels of control – one at protein degradation and the other at *de novo* transcriptional potential.

Since loss of E-cadherin and increased EGFR signalling are known markers of invasive mesenchymal cancer cells (Wong and Gumbiner, 2003), we asked if the E-cadherin re-expression and EGFR decreases were accompanied with another known epithelial cell marker. Cytokeratin 18 expression, a marker of mature epithelial cells, increased over the 6-day period in both DU-145 and PC-3 cell lines suggesting a reversion of mesenchymal phenotype characteristic of these cell lines (Figure 1A). This implied a generalised reversion to a more differentiated phenotype in the presence of hepatocytes.

Looking more closely at the subcellular localisation of this E-cadherin and EGFR expression, freshly isolated GFP-expressing primary rat hepatocytes were allowed to adhere 24 h before seeding of the RFP-expressing prostate cancer cells. As expected, immunofluorescence showed increases in E-cadherin and β-catenin and inversely related decreases in EGFR expression in the presence of hepatocytes. Unexpectedly however, early co-cultures revealed E-cadherin expression at the prostate cancer cell periphery juxtaposed to the hepatocytes during attachment (Figure 2A and B). β-Catenin was also localised to the membrane area in these cells further suggesting a functional E-cadherin linkage at the interface of these two cell types. To our knowledge, E-cadherin interaction among different cell types has not been noted previously, thus we consider these interactions as potentially heterotypic in terms of cell types. While various other proteins (e.g. selectins and integrins) have been implicated in carcinoma attachment, heterotypic cell–cell interaction via E-cadherin has not. Therefore, the ability of prostate cancer cells to utilise E-cadherin to bind to hepatocytes was tested by assessing cell–cell adhesion between adherent hepatocytes and prostate carcinoma cells using the centrifugal assay for cell adhesion (McClay *et al*, 1981). Both DU-145 and PC-3 cells, calcein-AM-labelled, bound to the hepatocytes to a limited but statistically significant degree, and this binding was eliminated by an E-cadherin blocking antibody (Figure 3A–E). As the cell–cell adhesion of the prostate carcinoma cells was limited, though real, we sought to increase the levels of E-cadherin on these cells. For this we used the EGFR kinase inhibitor, PD153035, that we had shown earlier to promote E-cadherin expression in DU-145 cells within 24–48 h (Yates *et al*, 2005), and confirmed in PC-3 cells at 48 h (Figure 3C). This 48-h pretreatment with PD153035 increased prostate carcinoma cell binding to the hepatocyte monolayer (Figure 3A–E).

The above findings implicate EGFR signalling as potentially negatively regulating E-cadherin levels. As this was reminiscent of the situation with LHRH receptor transmodulation of autocrine EGFR signalling in DU-145 cells (Yates *et al*, 2005), we asked whether a similar situation might be functioning in this setting. We expressed the PKC transmodulation-resistant EGFR A654 variant (Welsh *et al*, 1991) in the DU-145 cells. Co-culturing these cells with hepatocytes did not lead to decreases in EGFR or increases in E-cadherin and cytokeratin 18 expression (Figure 4). If anything, the DU-145 A654 cells exhibited a decrease in E-cadherin expression over 6-day period, which would be expected since

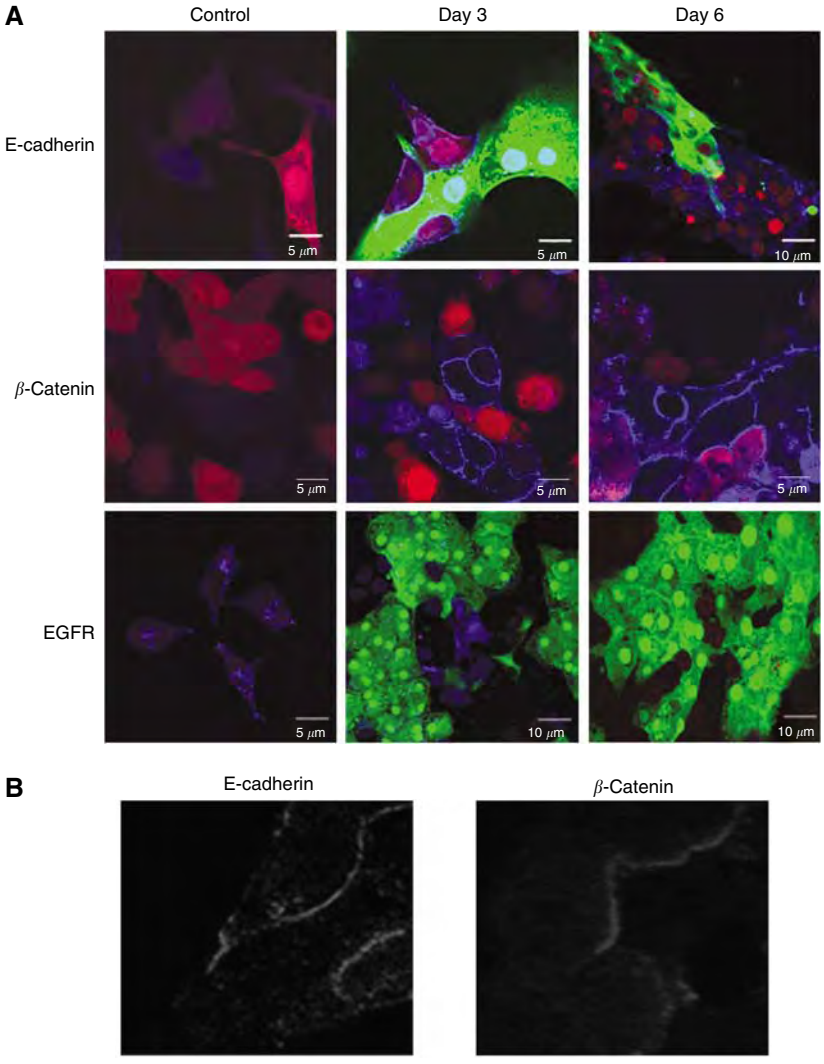

Figure 2 Immunofluorescence of co-cultures show subcellular location of E-cadherin re-expression. (**A**) Immunofluorescence of DU-145 RFP (red) and GFP (green) primary rat hepatocytes were stained with human-specific anti-E-cadherin, pan-species anti-β-catenin or human-specific anti-EGFR antibody. Cy5 secondary antibody (blue) was used for respective primary antibodies. Note the gain of blue (E-cadherin) in the RFP/red prostate cells in the top and middle rows, and the loss of blue (EGFR) in the bottom row. (**B**) The blue channel only of the lower left inset in the E-cadherin and β-catenin staining on day 2 is shown in black and white to demonstrate the localisation of the human E-cadherin in the prostate carcinoma cells to the interface with the hepatocytes. In the middle row, the hepatocytes were from WT and not GFP rats, so as not to interfere with the antibody staining as the anti-β-catenin detected both human and rat. However, the presence of β-catenin upregulation in the DU-145 cells is noted by a violet color and the membranous pattern at the hepatocyte–prostate carcinoma cell interface. Shown are representative of at least three experiments.

EGFR signalling continued unabated, and EGFR activity is well documented as leading to E-cadherin downregulation.

These data present a potential metastatic target organ-specific regulation of prostate carcinoma cell phenotype. However, to validate whether this may occur in *de novo* human tumours, we obtained human liver tissue from two patients with prostate cancer metastases to the liver and examined the expression of E-cadherin in these tumours by immunohistochemistry. E-cadherin staining was significant in the tumour nodules within the liver (Figure 5A). This increased expression was accompanied by increases in E-cadherin-associated adhesion molecules α, β and p120 catenin as well (Figure 5A). Central to our model of inverse relationship between E-cadherin expression and EGFR (Yates *et al*, 2005), we were able to observe a lack of total and active EGFR expression in these tumours (Figure 6A). To molecularly determine if these tumours reverted to a more epithelial phenotype, we stained for the epithelial marker cytokeratin 18 and the mesenchymal marker vimentin. Akin to our *in vitro* findings, these tumours largely express cytokeratin and lack vimentin expression (Figure 6B). In the absence of the primary tumours, from which these metastases derived, we cannot state that this represents a reversion in the phenotypic profile, but given the widespread finding of EMT in invasive and metastatic primary tumour, it does suggest that there may be cancer cell phenotypic variability as result of the metastatic microenvironment.

DISCUSSION

Loss of E-cadherin is so widely observed in advanced carcinomas that the E-cadherin molecule is considered a tumour suppressor (Wong and Gumbiner, 2003). This conception of E-cadherin is supported by forced re-expression of E-cadherin resulting in diminished tumourigenic potential in experimental tumour systems (Jawhari *et al*, 1999; Yates *et al*, 2005). However, unlike classical tumour suppressors (e.g. p53 and Rb), E-cadherin loss occurs by epigenetic downregulation or transcriptional silencing, rather than genetic deletion or mutation (Graff *et al*, 1995). As the

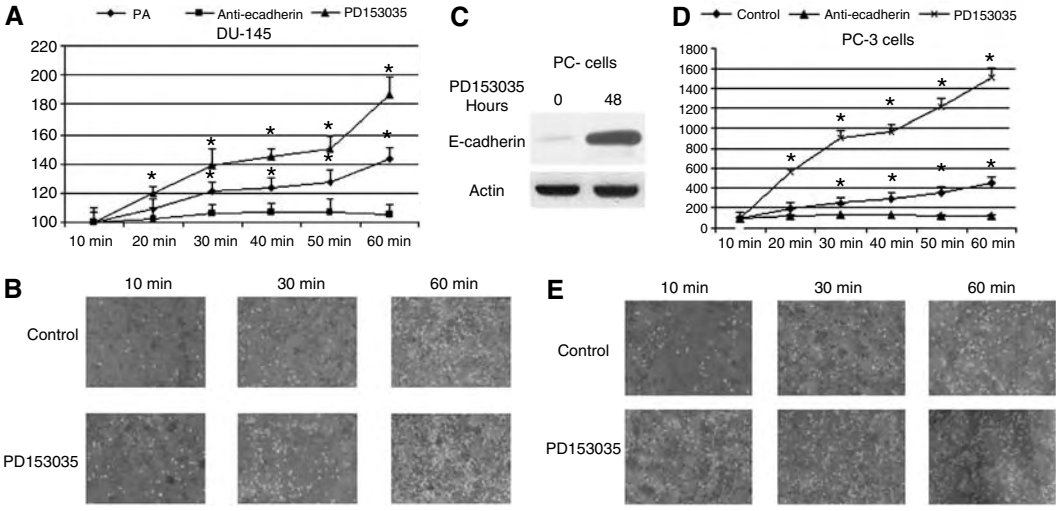

Figure 3 Human prostate cancer cells form E-cadherin-mediated heterotypic interactions with hepatocytes. DU-145 (**A** and **B**) cells or PC-3 (**C**–**E**) cells fluorescently labelled with Calcein A were incubated for 48 h with PD153035 or E-cadherin blocking antibody and seeded onto a monolayer of hepatocytes from non-GFP-expressing rats to analyse their ability to adhere. Cell binding was assessed by fluorescent intensity using a plate fluorometer and visually verified under a fluorescent light microscope. Y-axis is arbitrary fluorescent units. Data represent mean of three experiments performed in triplicate; s.e. $^*P < 0.05$. Shown are sample representative fields to show the bound tumour cells (converted to white dots) overlying the hepatocytes in **B** and **E**. (**C**) PC-3 cells were exposed to PD153035 for 48 h with a resultant upregulation of E-cadherin as shown by immunoblotting. This is similar to our previously published finding with DU145 cells (Yates *et al*, 2005).

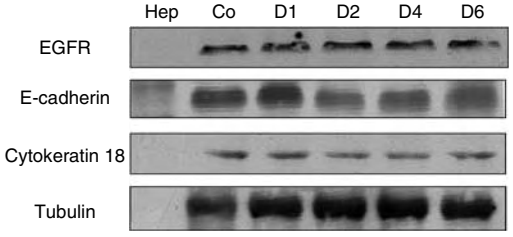

Figure 4 DU-145 cells expressing a PKC transattenuation-resistant EGFR (A654) are resistant to hepatocyte-induced E-cadherin re-expression. DU-145 A654 cells co-culture lysates were immunoblotted with an antibody selective for human E-cadherin, EGFR, cytokeratin 18 or tubulin. The legend is as with Figure 1, Co (control) DU145 A654 cells and Hep (hepatocytes) only. Shown is one of two representative blot series.

mechanisms which diminish E-cadherin are reversible, this opens the question as to whether there are situations in which re-expression of E-cadherin and subsequent cell–cell adhesion may promote tumour progression. Herein, we provide proof of principle that parenchymal cells from a tissue frequently targeted for prostate carcinoma metastasis, the liver (Ewing, 1922; Lind *et al*, 2004; Shah *et al*, 2004), signal this re-expression of E-cadherin.

Downregulation of E-cadherin occurs frequently in the primary prostate tumour coincident with aggression and spread (Gingrich *et al*, 1996; Kallakury *et al*, 2001). However, expression in distant metastases has been noted in some prostate cancer metastases (Rubin *et al*, 2001) but not in other studies in metastases to the bone (Bryden *et al*, 2002). Our findings herein, appear consistent with the re-expression, though the liver environment is likely substantially different from that of the bone in terms of signals to the hepatocytes and requirements by the metastatic tumour cells. It must also be noted that there is precedence for E-cadherin expression on disseminated carcinomas, as ovarian carcinoma dissemination throughout the peritoneal cavity shows increased levels of E-cadherin and its attendant catenins (Imai *et al*, 2004). Again, though, the applicability to metastatic spread of an organ-confined prostate cancer to a distant-defined organ compartment may not be comparable to the spread of ovarian carcinoma throughout an adjacent open cavity.

The findings herein raise two questions that need to be explored in depth in future investigations. First and most obvious, the mechanism by which the hepatocytes signal for E-cadherin re-expression needs to be defined. Our initial findings strongly suggest a link to downregulation of autocrine EGFR signalling. EGFR levels change inversely to the E-cadherin levels, and direct inhibition of EGFR signalling leads to increased E-cadherin expression (Yates *et al*, 2005). More directly, expression of an EGFR variant that is resistant to PKC-mediated attenuation renders the DU-145 cells impervious to hepatocyte-induced E-cadherin expression. This presages the ultimate issues of the nature of the signals from the hepatocytes and which carcinoma intracellular processes actuate the E-cadherin re-emergence. The intracellular events may be complex and involve both loss of downregulation at the protein level and reversion of transcriptional suppression. That EGFR activity is known to disrupt the E-cadherin/catenin complex leading to E-cadherin protein destabilisation and degradation (Ackland *et al*, 2003) and the impression that E-cadherin protein expression may precede increased mRNA suggest that protein downregulation is part of the answer. Although the intracellular mechanisms of E-cadherin re-expression are undoubtedly complex, these findings are novel and provide insight of prostate cancer cell behavior within a target soft organ.

One caveat that deserves specific mention is that EGFR signalling contributes to numerous aspects of prostate tumour progression. For instance, autocrine EGFR-mediated motility promotes tumour invasion and metastasis and tumour cell proliferation and survival (Wells, 2000). Thus, targeting EGFR kinase may have effects other than promoting the metastatic engraftment proposed herein by the upregulation of E-cadherin. For instance, the recent report of EGFR kinase inhibitors limiting prostate tumour dissemination in experimental models (Angelucci *et al*, 2006) is likely due to inhibiting pathways and cell behaviors before the proposed upregulation of E-cadherin at the metastatic site, though re-expression of E-cadherin at the primary tumour site would also limit spread by preventing initial detachment (Jawhari *et al*, 1999; Lowy *et al*, 2002; Wong and Gumbiner, 2003).

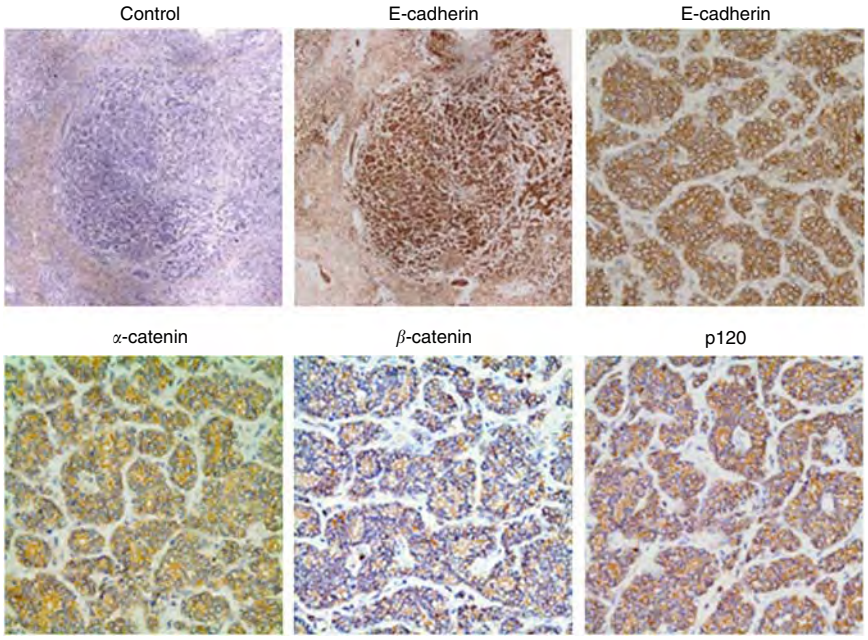

Figure 5 Human prostate cancer metastases to liver show expression of cell–cell adhesion molecules. Formalin-fixed, paraffin-embedded tissues were obtained from two well-defined prostate adenocarcinomas with liver metastasis. Tissues were stained with indicated antibodies, Secondary antibody, anti-mouse only as the staining control (top left; 3700 μm^2), anti-E-cadherin (top centre; 3700 μm^2 and top right; 300 μm^2), anti-α-catenin (bottom right; 300 μm^2), anti-β-catenin (bottom centre; 300 μm^2) and anti-p120 (bottom right; 300 μm^2). Shown are representative of repeated stainings; the other metastasis presented similar findings.

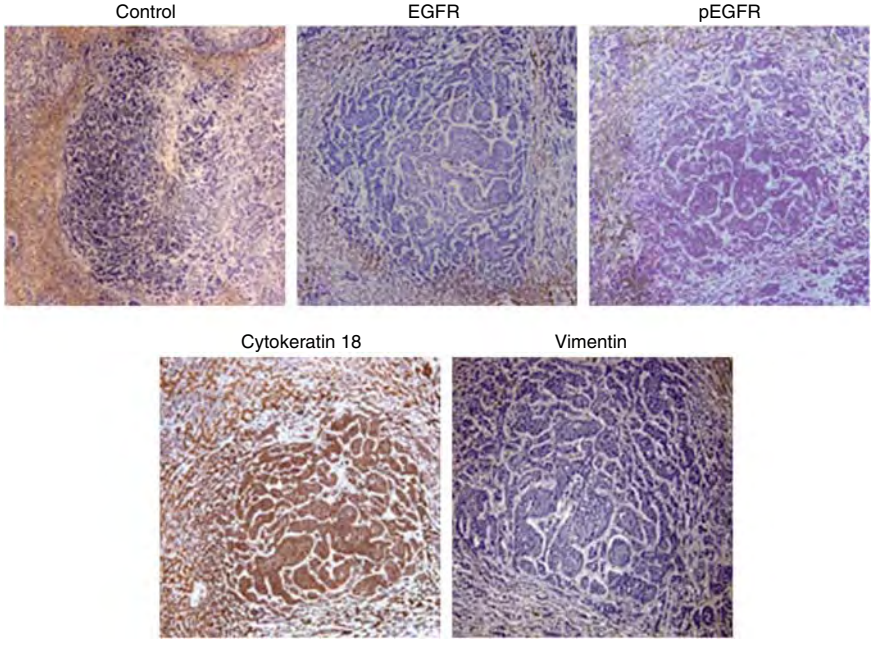

Figure 6 Human prostate cancer metastases show reversion of metastatic markers. Tissues were stained with anti-rabbit (top left; 1400 μm^2) anti-EGFR (top centre; 1400 μm^2), anti-phosphotyrosyl-EGFR (activated EGFR) (top right; 1400 μm^2), anti-vimentin (bottom left; 1400 μm^2) and anti-cytokeratin 18 (bottom left; 1400 μm^2). Shown are representative of repeated stainings; the other metastasis presented similar findings.

The second question of what selective advantage is conferred by E-cadherin re-expression remains in the speculative realm. Our finding and those reported in the literature (Rubin *et al*, 2001; Kowalski *et al*, 2003) or carcinoma metastases presenting E-cadherin and differentiation-related cytokeratins, along with low to absent levels of vimentin and EGFR (total and active), cannot discern whether these metastases arose from a subset of primary tumour cells that never underwent a phenotypic shift or from the majority of aggressive cells that underwent the so-called epithelial–mesenchymal transition. However, the fact that experimental prostate cancer cell lines, DU-145 and PC-3, exhibited similar phenotypic shifts when cultured with hepatocytes implies a survival or growth advantage in these ectopic sites that is at odds with that seen for the EMT tumour cells in the primary locale. Recently, this has been supported experimentally by the finding that epithelial variants of a bladder carcinoma cell line presented

greater seeding of metastatic sites when introduced into the circulation than the mesenchymal parental line, whereas the parental mesenchymal tumour line possessed increased metastatic capabilities from an orthotopic location (Chaffer *et al*, 2006). One can speculate that in a metastatic tumour microenvironment lacking many of the prostate-specific survival signals, E-cadherin-mediated linkages provide critical survival signals, by linking to both other carcinoma cells and even the hepatocytes themselves. Such studies, which lie beyond the realm of the current communication, are underway.

In conclusion, we propose a model in which the differentiation phenotype of metastatic prostate carcinoma cells is plastic in response to the microenvironment. In the primary tumour setting, E-cadherin expression is downregulated as part of the EMT that allows disaggregation from the primary mass enabling invasive migration and distant dissemination. However, once the tumour cells reach the ectopic microenvironment, numerous signals critical for survival and/or growth are absent. The metastatically competent subset of tumour cells interpret initiating signals from the target organ, in this case the liver, to re-express a more differentiated phenotype, or to undergo a mesenchymal–epithelial *reverse* transition. This redifferentiation could provide for both homotypic and heterotypic cell–cell adhesion with concomitant survival signals. While such a redifferentiation would be expected to limit tumour cell proliferation and local invasiveness, it may be key to prevent tumour cell apoptosis in the absence of a supportive orthotopic microenvironment. In-depth exploration is necessary not only to determine if such metastatic carcinoma redifferentiation occurs in *de novo* metastasis of human tumours, but also to explain the relative resistance to chemotherapy of metastases and even the concept of tumour dormancy.

ACKNOWLEDGEMENTS

We thank Linda Griffith and George Michalopoulos for discussions and suggestions. These studies were supported by grants from the VA Merit Award Program and the DoD Congressionally Directed Medical Research Program.

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